Reprinted from:
MICROBIAL TOXINS, VOL. 6

Academic Press, Inc., New York and London

CHAPTER 6

Patulin, Penicillic Acid, and Other Carcinogenic Lactones

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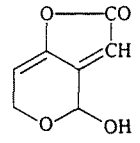
I. Patulin

A. INTRODUCTION

Patulin, 4-hydroxy-*4H*-furo[3,2c]pyran-2(*6H*)-one, has an empirical formula $C_7H_6O_4$ and molecular weight 154. It is a lactone metabolite of several species of *Penicillium* and *Aspergillus*: *Penicillium urticae*, *Penicillium claviforme*, *Penicillium expansum*, *Aspergillus clavatus*, *Aspergillus giganteus*, and *Aspergillus terreus*, and a species of *Byssochlamys nivea* (Shibata *et al.*, 1964).

Patulin is unstable in alkali and loses biological activity (Atkinson, 1942; (Chain *et al.*, 1942), but it is stable in acid (Chain *et al.*, 1942). Details of the history of discovery of patulin and the various names given to it—clavacin, claviformin, expansin, mycoin c, and penicidin—can be found in the reviews by Florey *et al.* (1949) and Singh (1966). The chemical structure of patulin was elucidated in 1949 by Woodward and Singh and is depicted in Fig. 1.



Patulin

FIG. 1. Structure of patulin.

B. TOXICITY

As shown in Table I, patulin has been found to be toxic at varying degrees to a number of microorganisms. Patulin inhibits the growth of both gram-positive and gram-negative bacteria. More than 75 bacterial species have been tested and none are completely resistant to the effects of patulin. However, not all fungi are susceptible to patulin; e.g., *A. clavatus* was not affected at a concentration of 1 mg/ml. The sensitivity of different species of protozoa is dependent on the concentration of the antibiotic and the time of incubation. For example, a complete kill of *Glaucoma pyriformis* requires 24 hours at 2 μg/ml, and only 1 hour at 200 μg/ml (Jirovec, 1949). Other species, such as those of *Strigomonas*, are more resistant and require an exposure time of 24 hours at 200 μg/ml for a total kill.

This antibiotic is also most effective in inhibiting the growth of tissue cultures. Patulin caused a 50% reduction in multiplication of mouse fibroblasts in suspension cultures (Perlman *et al.*, 1959; A. R. Powell, 1966). Vollmar (1947) has reported that mouse leukocytes and normal epithelium cultures from rabbit cornea were stimulated at concentrations of 20-40 μg/ml and inhibited at concentrations of 100-200 μg/ml. Patulin has also been tested against influenza virus in mice inoculated with that virus; however, its use proved to be rather ineffective (Rubin, 1947-1948). High concentrations of patulin have been effective in inactivating *Escherichia coli* bacteriophages (D. Jones, 1945) and many other bacterial viruses (Hall *et al.*, 1951); however, low concentrations of patulin were found to be ineffective with phages of *Pseudomonas* sp. (Dickenson, 1948).

The phytotoxic activity of patulin has also been evaluated in a number of plant systems. This metabolite was found to be inhibitory to seed germination (Gattani, 1957; Timonin, 1946; Wallen and Skolko, 1951) and responsible for plant wilting (Gäumann and Jaag, 1947; Iyengar and Starkey, 1953; Nickell and Finlay, 1954; Wang, 1948; Wright, 1955). Ark and Thompson (1957) have shown that patulin (100 ppm) gives a complete inhibition of germination of sporangia of *Pseudoperonospora cub-*

ensis in *in vitro* tests. Anslow *et al.* (1943) have demonstrated complete inhibition of growth of various species of *Pythium* (the cause of damping-off disease of seedlings) at dilutions of 1:400,000 patulin. Patulin is also a strong inhibitor of plasma streaming in Elodea canadensis (Gäumann and Jaag, 1947) and in *Solanum lycopersicum* (Miescher, 1950). This effect can be blocked by sulfhydryl compounds in tomato sprouts (Miescher, 1950).

Toxicity in animals has been demonstrated by numerous investigators (Broom *et al.*, 1944; Chain *et al.*, 1942; Schweitzer, 1946). In 1945, Oosterhuis (1945-1947) demonstrated that the application of patulin as an ointment was useful for treating clinical dermatomycosis. Bollag (1949)

TABLE I
TOXICITY OF PATULIN TO MICROORGANISMS

Microorganism	Concentration inhibitory to growth (μg/ml)	Reference
Protozoa		
Euglena gracilis	100	1
Paramecium auvelia	10	2
Trypanosoma equiperdon	0.10	3
Fungi		
Candida albicans	300	4
Penicillium nigricans	62.5	5
Ustilago tritici	10	6
Actinomycetes		
Actinomyces scabies	6.25	7
Bacteria		
Agrobacterium tumefaciens	2	8
Escherichia coli	6-10	9
Bacillus subtilis	12.5-100	10
Micrococcus pyogenes	8	10
Staphylococcus aureus	12.5-30	11
Cells and tissue cultures		
Ehrlich carcinoma	20-40	12
Leukocytes	1.25	13
Chick fibroblasts	100	14
Chick heart cultures	10	15
Mouse ascites tumor cells	60	16

Key to references: 1. Jirovec (1949); 2. Austin *et al.* (1956); 3. Rubin (1947-1948); 4. Reilly *et al.* (1945); 5. Wright (1955); 6. Timonin (1946); 7. Gilliver (1946); 8. Klemmer *et al.* (1955); 9. DeRosnay *et al.* (1952); 10. Kavanagh (1947b); 11. Raistrick *et al.* (1943); 12. Vollmar (1947); 13. Chain *et al.* (1942); 14. Broom *et al.* (1944); 15. Keilova-Rodova (1949); 16. Lettré *et al.* (1954).

has investigated the effect of patulin upon mice, observing that the lymphocyte count decreased markedly in the blood after the administration of 100 μg/day, but the granulocyte count was normal. After termination of injections, the lymphocyte count increased again and was accompanied by an increase in granulocytes. The pharmacological studies with this antibiotic have shown that patulin increases vascular permeability, causing serious edema. Katzman *et al.* (1944) have also shown that patulin is highly effective in suppressing the formation of urine in mice and causing an increase in blood sugar. The LD_{50} (intravenous) for mice and rats is approximately 0.3-0.7 mg/20 gm body weight (Broom *et al.*, 1944). Boyd in 1944 had shown that when patulin was used in concentrations of 1:20,000 as a nose and throat spray it was effective in treating common head colds. Patulin also has a spasmolytic effect, as demonstrated by the inhibition of induction of contractions by guinea pig ileum preparations at 3-10 μg/ml (Eliasson, 1958). Vick (1959), studying the physiological properties of isolated, perfused guinea pig ventricle, showed that high concentration of patulin caused a brief increase in contractions.

C. Cellular Effects

Patulin inhibits cell and/or nuclear division, but the observations regarding mechanism of action are not in agreement. Babudieri (1948) reported that patulin inhibited fission in bacteria, yielding giant cells. In 1948 Wang reported that patulin treatment yielded binucleate cells in corn and onion roots; however, no chromosomal aberration was noted. Keilova-Rodova (1949), studying fibroblast and osteoblast cultures of chick embryos, demonstrated that patulin administration resulted in mitostatic (inhibition of mitosis) action with the chromosomes in a pathological condition at all stages of mitosis, especially at metaphase. This investigator observed a reduction in the number of prophase chromosomes and frequent formation of binucleate cells. Steinegger and Leupi (1956) reported that patulin has a mitostatic action upon the chromosomes and nucleus of *Allium cepa* and *Lepidium sativum* and believed that the spindle mechanism was not affected. However, Rondanelli *et al.* (1957) demonstrated that the sulfhydryl compounds glutathione and cysteine prevented the mitostatic effect of patulin and concluded that the spindle mechanism was affected via the reaction of patulin with sulfhydryl groups of the contractile fibers, rendering them inelastic. The antibiotic also induces alterations of the mitotic spindle and fragmentation of the chromosomes in the segmenting eggs of salamanders (Sentein, 1955). The toxic action of patulin can also be inactivated by thioglycolate and dimercaptopropanol (Atkinson and Stanley, 1943a,b; Burton, 1949; Bustinza and

Lopey, 1949; Cavallito *et al.*, 1945; Delavnay *et al.*, 1955; Geiger and Conn, 1945).

The antibiotic activity of patulin is decreased by substances such as methionine, asparagine, and peptone (DeRosnay *et al.*, 1952; Waksman and Bugie, 1943), and sodium sulfite and pyrosulfate (Boyd, 1944). However, tryptophan, urea, and thiourea increase the toxic effects of patulin (DeRosnay *et al.*, 1952).

Dickens (1967) and Dickens and Jones (1961) have reported that patulin administered subcutaneously twice weekly to rats for approximately 15 months produced malignant tumors at the injection site. Patulin has been implicated in abnormal developments in the cell nucleus, including total or partial fragmentation of chromosomes. It has been suggested that many of the cytological alterations are the direct result of initial inhibition of the respiratory process (Singh, 1967). Ascladiol, a reduction product of patulin, has been isolated from *A. clavatus* (Tanabe and Suzuki, (1970). The toxicity of the substance is about one-fourth that of the parent molecule patulin.

Lettré *et al.* (1954), characterizing tumor inhibitors by use of tagged tumor cells (mouse ascites tumor cells with ^{32}P), showed that 100 μg patulin increased the tumor-cell doubling time from 1.2-2.0 to 4.7-5.5 days. Another report by Hannig (1961) shows that patulin inhibits the proliferation of cancer cells.

D. Biosynthesis and Biochemical Effects

The biosynthetic scheme for patulin from glucose-^{14}C has been studied extensively by Bu'Lock and Ryan (1958) and is depicted in Fig. 2. The transformation of biosynthetically labeled 6-methylsalicylate (6-MS) (from acetate-1-^{14}C) into patulin was found to occur by Bu'Lock and Ryan (1958). The pattern of isotopic distribution in this compound agreed quite well with the oxidative molecular rearrangement proposed for patulin biosynthesis.

Numerous investigations have been carried out in determining the mode of action of this compound and the biochemical effects it elicits in microbial and plant systems. Patulin inhibits the aerobic respiration of bacteria (Gottlieb and Singh, 1964; Lembke and Frahm, 1947; Lembke and Hahn, 1954), fungi (Gottlieb and Singh, 1964), and guinea pig kidney slices and brain homogenates (Andraud *et al.*, 1963). This compound partially inhibited cocarboxylase at 5.2×10^{-2} *M* (Karrer and Viscontini, 1947). Dehydrogenase activities have also been inhibited in mouse ascite tumor cells at 20 μg patulin/ml (Holscher, 1951); however, Schmitz (1950) found no such inhibition at 30-120 μg/ml using the Thunberg tech-

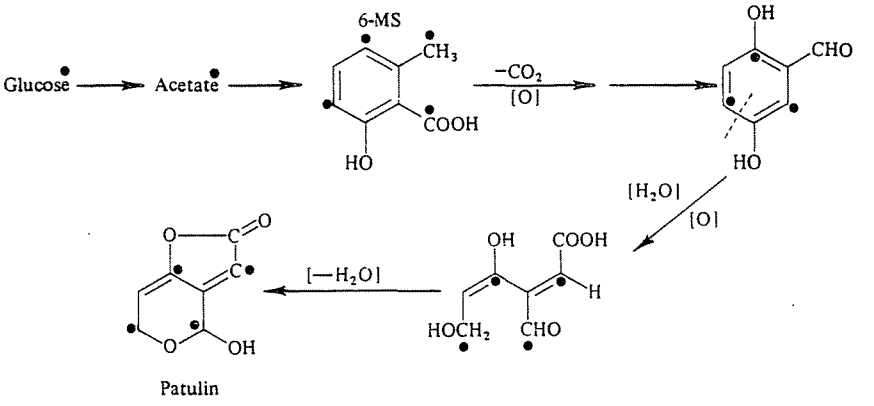


FIG. 2. Biosynthesis of patulin (Bu'lock and Ryan, 1958). Dot indicates carbon derived from acetate carboxyl.

nique. Succinate oxidase and succinate dehydrogenase activities were strongly inhibited (90%) at 5×10^{-2} *M* in cell-free extracts (Gottlieb and Singh, 1964).

The inhibitory effect of patulin has been attributed to blockage of respiration. Inhibition may occur indirectly as a result of the effect on cellular membrane permeability. This alteration may allow leakage of vital metabolites out of the cell or inhibit nutrient transport into the cell, therefore arresting growth and respiration. Patulin also inhibits uptake of K^+ ions in erythrocytes (Kahn, 1957) and glucose in fungal mycelium (Singh, 1967).

II. Penicillic Acid

A. Introduction

Penicillic acid was first isolated by Alsberg and Black in 1913 from *Penicillium puberulum* grown on corn. Even at this early date, investigators were conjecturing on the possible toxicity of mold products (today called mycotoxins) to animals. Alsberg and Black (1913) cite work by an Italian investigator, Gosio, who reported in 1896 that the culture medium on which a *Penicillium* had been grown was toxic to various laboratory animals. Gosio had isolated a crystalline compound having the empirical formula $C_9H_{10}O_3$ from the supernate, a formula remarkably similar to that of penicillic acid, $C_8H_{10}O_4$. Other investigators during this period also noted that various molds were capable of producing toxic substances on corn. Unfortunately, fungal taxonomy in the early 1900's was in its infancy, so that the identity of most of the molds studied in these early toxi-

cological investigations cannot be established. The fungus used by Alsberg and Black had been isolated originally from corn by F. D. Heald and subsequently identified by C. Thom of the United States Department of Agriculture (USDA). This culture is now listed in the Agricultural Research Service (ARS) Culture Collection of the USDA as *P. puberulum* NRRL 1889.

Alsberg and Black (1913) established that penicillic acid was toxic to various laboratory animals and conjectured that fungal toxins might be involved in pellagra resulting from consumption of moldy corn. Birkinshaw and Raistrick (1932) confirmed the toxicity findings of Alsberg and Black, but production of penicillic acid by their culture was too low to permit additional study. Birkinshaw and his colleagues (1936) later found that *Penicillium cyclopium* Westling produced relatively large amounts of penicillic acid, and they were able to characterize the molecule both as to structure and chemical properties.

B. Chemical Properties

Structure of the anhydrous penicillic acid (Fig. 3) can exist in two tautomeric forms—as a substituted γ-keto acid (γ-keto-β-methoxy-δ-methylene-A^{α}-hexenoic acid) or as a γ-hydroxylactone. Birkinshaw *et al.* (1936) cited evidence for existence of the keto form as (a) facile esterification of anhydrous penicillic acid with diazomethane and (b) ready reaction of penicillic acid with one molecule of free hydroxylamine in the cold in aqueous solution. Evidence for the lactone was found by production of a neutral acetyl derivative from the action of acetic anhydride on penicillic acid. The two tautomeric forms were studied in depth by Szilágyi *et al.* (1963). Penicillic acid has a molecular weight of 170.16 (C, 56.46%; H, 5.93%; O, 37.61%) and crystallizes as anhydrous needles from pentane,

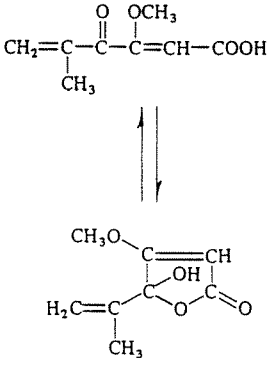


FIG. 3. Structure of penicillic acid.

hexane, or benzene with a mp 83-84.8°C. The monohydrate ($C_8H_{12}O_5$) crystallizes from water as large transparent monoclinic, or triclinic, rhombic crystals (mp 58-64°C).

Penicillic acid is moderately soluble (2%) in cold water and in cold benzene, highly soluble in hot water, alcohol, ether, and chloroform, and insoluble in pentane-hexane.

Using infrared spectral data, Kovac and co-workers (1969) were able to demonstrate that penicillic acid strongly self-associates, yielding mainly a dimer of penicillic acid.

Penicillic acid was chemically synthesized by Raphael in 1947. Various benzene derivatives structurally similar to penicillic acid were synthesized to determine the extent and type of contribution made by the various moieties of the penicillic acid molecule to its antibacterial properties (Amstutz *et al.*, 1946). Additional derivatives (2-hydroxy-2-phenyl-3-methoxy-2,5-dihydrofuranone, $C_{11}H_{12}O_4$, and 2-hydroxy-2-phenyl-3-phenoxy-2,5-dihydrofuranone, $C_{16}H_{12}O_4$) were synthesized by starting from β-bromo-β-benzoylacrylic acid and substituting the bromine with a methoxy or phenoxy group (Zugrăvescu *et al.*, 1957). Penicillic acid can be complexed with potassium siliconate (Gueyne and Duffant, 1962).

C. Analysis

Gravimetric, colorimetric, and fluorodensitometric methods of analysis have been described for penicillic acid. Birkinshaw and Gowlland (1962) extracted a culture liquor after acidification with ether, then removed the solvent, and weighed the residual solids. A given weight was fractionally sublimed *in vacuo* (penicillic acid sublimes at 80-90°C) and the sublimate was weighed.

Penicillic acid reacts with hydroxylamine in strong alkali to give a red color (Sternberg, 1956). The absorption maximum of the complex is at 530 mμ, and Beer's law is followed between 80 and 1000 μg/ml. The color is stable and not influenced by light, temperature, or inorganic ions. Acetylpenicillic acid also gives the red color; however, dibromopenicillic does not. Bentley and Keil (1962) found that penicillic acid forms a stable reddish-purple complex with ammonia. The absorption maximum is at 545 mμ and the assay follows Beer's law between 200 and 2000 μg.

A more sensitive assay (1-10 μg) has been developed by Ciegler and Kurtzman (1970) based on the formation of a fluorescent compound after exposure of penicillic acid on a thin-layer chromatographic plate to ammonia vapor. One to ten micrograms penicillic acid was spotted on a silica gel plate (GHR), which was developed in ethyl acetate:chloroform:formic acid (40:60:1 v/v/v), air dried, and then exposed to ammonia vapor for 3

minutes. The fluorescence was measured densitometrically, and the amount of penicillic acid present was determined from a standard curve, Beer's law being followed between 1 and 10 μg penicillic acid. The fluorescent compound exhibited an excitation maximum at 350 mμ and an emission band at 440 mμ.

D. Biochemistry

Birch *et al.* (1958) studied the biosynthesis of penicillic acid by *P. cyclopium* using acetate-1-^{14}C as a precursor. The data suggested a head-to-tail condensation of four acetate units to yield orsellinic acid with subsequent ring cleavage to give penicillic acid. Mosbach (1960) demonstrated a similar sequence (acetate → orsellinic acid → penicillic acid) in *Penicillium baarnense*. Subsequently, it was shown that orsellinic acid was formed by the condensation of one molecule of acetyl coenzyme A and three of malonyl coenzyme A with the loss of three molecules of carbon dioxide (Bentley and Keil, 1961). The *O*-methyl group at C-8 is introduced at some unknown point in the biosynthetic sequence; formate and the methyl group of methionine are efficient precursors for this methyl group. Glucose-1- and 6-^{14}C gave a labeling pattern similar to that from acetate-2-^{14}C. Carbons 2, 4, 6, and 7 are derived from the C-1 or C-6 of glucose and from the methyl group of acetate, carbons 1, 3, and 5 from the carboxyl group of acetate. Methylene- and carboxyl-labeled malonates were also converted into penicillic acid (Fig. 4). A detailed tracer study of penicillic acid biosynthesis by *P. cyclopium* was made by Bentley and Keil (1961) using glucose and various intermediates such as acetate and malonate. Their data provided further evidence for the head-to-tail condensation of a single acetate unit with multiple units of malonate; mevalonate had been previously ruled out as an intermediate for penicillic acid synthesis (Birch *et al.*, 1958). Birch *et al.* (1958) had earlier suggested that orsellinic acid was cleaved to give penicillic acid in a manner similar to that indicated for the conversion of gentisic acid to patulin (Bassett and Tanenbaum, 1958). The known data to date summarizing the biosynthesis of penicillic acid are shown in Fig. 5.

E. Biological Properties

Penicillic acid is synthesized by a large number of fungi: *P. puberulum*, *Penicillium stoloniferum* (Alsberg and Black, 1913); *P. cyclopium* (Birkinshaw *et al.*, 1936); *Penicillium martensii* (Wirth *et al.*, 1956); *Penicillium thomii*, *Penicillium suavolens* (Karow *et al.*, 1944); *Penicillium*

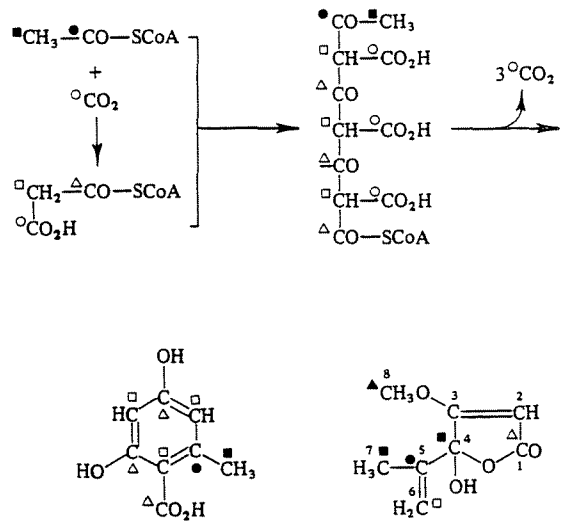


FIG. 4. Derivation of the carbon atoms in penicillic acid (Bentley and Keil, 1961). ●, carbon derived from malonate carboxyl or carbon dioxide; ■, carbon derived from C-1 of C-6 of glucose, or from acetate methyl; ●, carbon derived from acetate carboxyl; □, carbon derived from C-1 of C-6 of glucose, from acetate methyl, or from malonate methylene; △, carbon derived from acetate or malonate carboxyl. ▲, carbon derived from methyl of methionine or from formate.

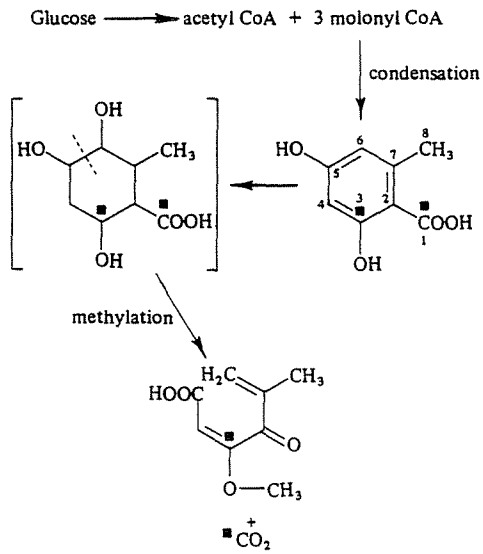


FIG. 5. Biosynthesis of penicillic acid.

palitans (Ciegler and Pitt, 1970); *P. baarnense* (Burton, 1949); *Penicillium madriti* (Birkinshaw and Gowlland, 1962); *Aspergillus ochraceus* (Karow *et al.*, 1944); *Aspergillus sulphureus* (Gill-Carey, 1949); *Aspergillus quercinus*, *Aspergillus melleus* (Burton, 1949). The wide distribution of this secondary metabolite indicates that its synthesis has little or no taxonomic significance.

Factors affecting penicillic acid production by fermentation or its occurrence in commodities have not been extensively studied. Alsberg and Black (1913) indicated that aeration and medium components affected production but presented no quantitative data. Birkinshaw *et al.* (1936) noted that their culture of *P. cyclopium* (L.S.H.T.M. Catalogue No. P123) produced no penicillic acid when grown on Czapek-Dox medium with glucose as the sole carbon source and sodium nitrate as the nitrogen source; however, this strain produced considerable quantities of penicillic acid in Raulin-Thom medium. Udagawa *et al.* (1970) noted that several strains of *A. ochraceus* growing on a sucrose-glutamic acid-salts medium produced 0.7 gm/liter penicillic acid.

The antimicrobial spectrum has been investigated by Oxford and his colleagues (Oxford, 1942: Oxford *et al.*, 1942). The compound appears to be active primarily against gram-negative bacteria, although it is also active against some gram-positive species (Heatley and Philpot, 1947; Kavanagh, 1947a). It has very limited antihelminthic properties (Baciková *et al.*, 1965) but does affect the growth of oat seed coleoptiles by interference with the respiratory process (Bastin and van Roey, 1954). Penicillic acid has proven to be too toxic for use in therapy. The LD_{50} (subcutaneous) for mice is 100 mg/kg (Spector, 1957).

Dickens and his colleagues have studied extensively the carcinogenic action of lactones and related substances. Dickens (1962, 1967) and Dickens and Jones (1961) observed that subcutaneous injection of 1.0 mg/dose twice weekly produced transplantable tumors after 64 weeks in all rats surviving treatment. In addition, a dose as low as 0.1 mg initiated tumor development (Dickens and Jones, 1963a). Dickens (1964) pointed out in a study including carcinogenic five-membered ring systems that for maximum carcinogenic activity, it is desirable to have α,β-unsaturation, preferably together with the presence of an external conjugated double bond attached to the 4 position of the γ-lactone ring; this type of structure occurs in patulin and penicillic acid.

Dickens and Cooke (1965) attempted to correlate the rates of hydrolysis and rates of chemical interactions with the sulfydryl group of cysteine with the relative carcinogenicity of 47 lactones, including penicillic acid. The latter compound reacted rapidly with cysteine, but the authors found

that the rates of hydrolysis bore no simple relationship to the carcinogenic properties of lactones.

In view of the toxic properties of penicillic acid, it is rather curious that a patent was allowed in which this compound is added as a supplement to animal feed (Schröder, 1968).

F. Natural Occurrence

Blue-eye diseased corn is known to be extensively fed by farmers to livestock. Blue-eye disease of corn can be attributed to various fungi. Kurtzman and Ciegler (1970) isolated several strains of *P. martensii* from high-moisture corn stored at 5°C; the corn had been described as blue-eye diseased. Feeding this moldy corn to mice resulted in death. Kurtzman and Ciegler isolated, crystallized, and identified the toxic factor as penicillic acid. This compound was produced between 5° and 32°C with maximal production between 15° and 20°C. There was a sharp decrease in production at 25°C and above, with no mold growth occurring at temperatures above 32°C. These findings plus those of Dickens and his colleagues indicate that penicillic acid should be considered as a potential mycotoxin.

III Other Carcinogenic Lactones

A. Lactones As Alkylating Agents

It has been clearly established that compounds containing a lactone possess a wide range of pharmacological and cytotoxic properties (Dickens, 1967; Haynes, 1948; Kupchan *et al.*, 1968). In addition, the extensive studies of Dickens, Jones, and co-workers (Dickens, 1964, 1967; Dickens and Cooke, 1965; Dickens and Jones, 1961, 1963a,b, 1965; Dickens *et al.*, 1966) have shown that a wide variety of lactones are carcinogenic. Since many lactones are subject to nucleophilic attack, the biological activity of the compounds has been attributed to their action as alkylating agents (Dickens, 1964; Goldschmidt, 1965). Alkylation of nucleic acids, particularly the N-7 position of guaninine, has been implicated as the site of action of the lactones in susceptible cells (Roberts and Warwick, 1963). The alkylation of sulfhydryl groups has also been suggested as the mechanism of toxic action of these compounds (Harrington, 1967). Some carcinogenic lactones react *in vitro* with thiosulfate ion and cysteine (Dickens and Cooke, 1965; Goldschmidt, 1965; Van Duuren and Goldschmidt, 1966). However, there is little correlation between the rate of

thiol disappearance and carcinogenicity of several lactones, including aflatoxin. Therefore, J. B. Jones and Young (1968) have criticized tests evaluating carcinogenicity on the basis of interaction with cysteine. Since their results demonstrate that several carcinogenic lactones undergo attack resulting in the alkylation of a nucleophile while the inactive lactones yield acyl products, they propose a chemical evaluation of cancer-initiating compounds based on alkylation-acylation differences.

Although there may be no widely accepted chemical test for evaluating carcinogens, the role of sulfhydryl groups in the biological development of neoplasms has presented oncologists with many provocative working hypotheses. Harrington's review (1967) of the sulfhydryl-cancer literature is climaxed by a cancer-induction proposal involving interaction of a carcinogen directly with the sulfhydryl groups that function in the control of cell division. Szent-Györgyi *et al.* (1967) have presented a fascinating concept of tumor induction based on modification of critical sulfhydryl groups by glyoxal derivatives. Although *in vivo* evidence for a sulfhydryl-aflatoxin interaction is lacking, Smith (1963, 1965) has observed that aflatoxin inhibition of amino acid activating *in vitro* enzymes from liver and *E. coli* can be reversed by addition of cysteine or glutathione.

B. Five-Membered Carcinogenic Lactones

Since patulin and penicillic acid in the lactone forms contain carcinogenic five-membered ring systems (Figs. 1 and 3), this group of compounds has received attention as potential inducers of cancer (Dickens, 1967; Dickens and Jones, 1961, 1965; Dickens *et al.*, 1966). Penicillic acid lactone is included in the group of five-membered compounds referred to as tetronic acids, some of which are carcinogenic. For example, α-methyltetronic acid induces tumors in rats (Table II) following subcutaneous administration (2 mg/injection) of the compound (Dickens and Jones, 1965). This fact assumes some significance when structural comparisons are made with tetronic acids of fungal origin such as γ-methyltetronic acid (Fig. 6) (Clutterbuck *et al.*, 1935; Shibata *et al.*, 1964). Several such compounds have been isolated from a broad spectrum of molds (Evans *et al.*, 1969), although the bulk of the chemical characterization work has been done with metabolites of *Penicillium charlesii* (Shibata *et al.*, 1964). Although the carcinogenic five-membered substituted maleic anhydride (Fig. 6, Table II) has no direct structural analogs occurring as mold metabolites, several interesting compounds of fungal origin (Shibata *et al.*, 1964) do include the anhydride group (Fig. 6) such as rubratoxin (a hepatoxin from *Penicillium rubrum*) (Moss *et al.*, 1968), puberulonic acid (*Penicillium* spp.) (Birkinshaw and Raistrick, 1932), stipitatonic acid

TABLE II
THE CARCINOGENIC ACTION OF LACTONES AND RELATED SUBSTANCES BIOASSAYED BY SUBCUTANEOUS INJECTION TWICE WEEKLY[a]

Compound injected	Dose (mg/injection)	Treatment time (weeks)	Cancer: earliest appearance (weeks)	Rats with local tumors/ survivors
Aflatoxin ($B_1 + G_1$)	0.01	41	24	6/6
β-Propiolactone	0.1	34	25	4/4
Penicillic acid	1.0	64	48	4/4
α,β,-Dimethyl maleic anhydride	2.0	65	76	3/5
α-Methyltetronic acid	2.0	65	63	2/4
Penicillin G (sodium salt)	2.0	46-78	59	7/19

[a]From Dickens (1967), Dickens and Jones (1961, 1963a), and Dickens *et al.* (1966).

(*Penicillium* sp.) (Segal, 1957), glauconic and glaucanic acid (*Penicillium-Aspergillus* spp.) (Ferguson *et al.*, 1962), byssochlamic acid (*Byssochlamys fulva*) (Raistrick and Smith, 1933), and itaconitin (*Aspergillus* sp.) (Nakajima *et al.*, 1964).

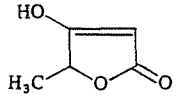
Dickens and Jones (1963a, 1965) have also compared the carcinogenicity of several compounds that structurally resemble maleic anhydride. Of these, succinic anhydride and maleic hydrazide (Fig. 6) showed approximately the same capacity to induce tumors as the substituted maleic anhydride. Although previous observations had shown that a double bond in the α,β-position to the lactone was consistently related to tumor-initiating capacities, the results with succinic anhydride revealed that unsaturation was not a structural requirement of carcinogenic compounds. Phenyl vinyl ketone, vinylene carbonate, β-angelica lactone, and sarkomycin also induced formation of tumors in test animals.

Sarkomycin (Fig. 6) is a product of *Streptomyces erythrochromogenes* possessing both antitumor and antibiotic activity (Umezawa *et al.*, 1954). Although the compound does not contain a lactone, it does possess a cyclopentanone ring having a carbonyl conjugated with an external methylene group; the structure is similar to other known carcinogens. Following subcutaneous injection of sarkomycin twice weekly (2 mg/dose) into rats, a transplantable myxosarcoma developed after 42 weeks.

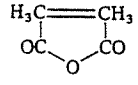
Yates *et al.* (1968) characterized a five-membered lactone resembling penicillic acid, a butenolide, from a culture of *Fusarium nivale* (Fig. 6). It

is toxic with an LD_{50} (intraperitoneal) of 44 mg/kg in mice and produces a toxic response when applied to rabbit skin. This compound may be involved in a potential mycotoxicosis, fescue foot, occurring in states such as Kentucky and Tennessee.

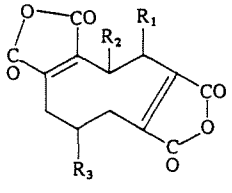
Clarke and Nord (1955) have described a product of *Stemphylium radicinum*, radicinin, which is structurally similar to patulin. Radicinin contains an α,β-unsaturated five-membered lactone fused to a six-membered ring containing oxygen. Although the nucleus of the structure is analo-

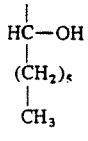
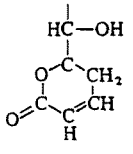


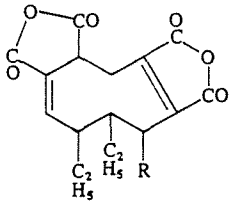
γ-Methyltetronic acid



α, β- Dimethylmaleic anhydride

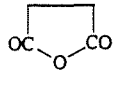


	R_1	R_2	R_3
Byssochlamic acid	C_3H_7	H,H	C_2H_5
Rubratoxin B	OH		

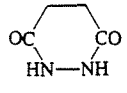


Glauconic acid, R = OH

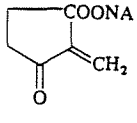
Glaucanic acid, R = H



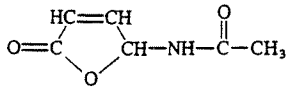
Succinic anhydride



Maleic hydrazide



Sarkomycin



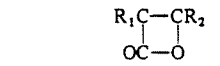
Butenolide

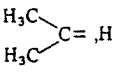
FIG. 6. Five-membered carcinogenic lactones and related structures.

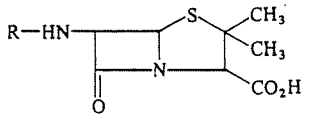
gous to that of patulin, there are side chain differences. Several species of *Monascus* also elaborate metabolites containing five-membered lactones fused to six-membered rings, including rubropunctatin, monascorubrin, and monascin (Shibata *et al.*, 1964). Strains of *Penicillium canadense* and *Aspergillus indicus* produce candensolide, a compound containing two fused five-membered lactone functions (Birch *et al.*, 1968; McCorkindale *et al.*, 1968). This substance is biologically active, demonstrating antifungal activity.

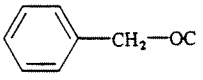
C. Four-Membered Carcinogenic Lactones

In addition to the carcinogenic properties of five-membered rings, English workers have also aroused interest in four-membered rings as tumor initiators (Dickens, 1964, 1967). They have shown that β-propiolactone (Fig. 7) and dimethyltrimethylene oxide are carcinogenic. β-Propiolactone initiates tumors following subcutaneous injections, repeated application to mouse skin, or by intratracheal intubation (Dickens and Jones, 1963a; Dickens *et al.*, 1966; Palmes *et al.*, 1962; Roe and Glendenning, 1956). Hydrolysis of the lactone prior to administration results in loss of



	R_1	R_2
β-Propiolactone	H,H	H,H
β-Butyrolactone	CH_3,H	H,H
2,2,4-Trimethyl-3-hydroxy-3-pentenoic acid-β-lactone		CH_3H



Penicillin G, R = 

6-Aminopenicillanic acid, R = H

FIG. 7. Carcinogenic four-membered ring structures.

carcinogenic activity (Dickens and Jones, 1963a). The four-membered lactone readily alkylates sulfhydryl groups *in vitro* forming a thio ether (*S*-2-carboxyethylcysteine) with cysteine (Dickens and Jones, 1961). In comparative tests of tumor initiation by β-propiolactone, penicillic acid, and aflatoxin, rats were injected subcutaneously with the compounds twice weekly until tumors developed (Table II) (Dickens and Jones, 1965). Dosing with 100 μg β-propiolactone, 1 mg penicillic acid, and 10 μg aflatoxin in each treatment, the time elapsed before the earliest appearance of tumors was 25, 48, and 24 weeks, respectively. Thus, aflatoxin was at least as effective a carcinogen as β-propiolactone, whereas penicillic acid was somewhat less active in these tests.

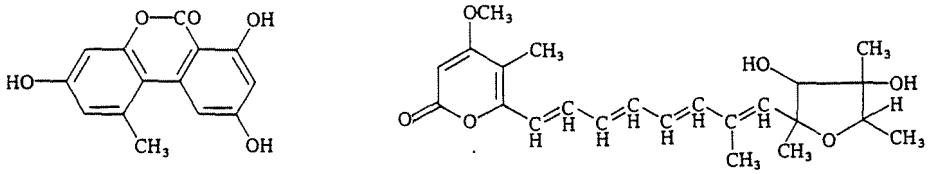
The presence of a four-membered lactam structure in penicillin led to examination of that fungal metabolite as a tumor initiator (Fig. 7). The significance of the lactam in biological activity of penicillin is apparent, since cleavage of the four-membered ring removes antibiotic activity of the compound (Dickens, 1967; Dickens and Cooke, 1965). Subcutaneous injection of 2 mg penicillin G (sodium salt) into rats twice weekly initiated tumor formation in two of the eight animals (earliest, 59 weeks) in an initial trial and five of eleven animals (earliest, 78 weeks) in a second test (Dickens and Jones, 1961, 1963a). Dose levels of 2 mg of penicillin G correspond to 3340 international units. Experiments were also carried out by Dickens and Jones (1965) determining the effect of repeated subcutaneous treatment in rats of 2 mg doses of the mold metabolite 6-aminopenicillanic acid (Fig. 7) (Batchelor *et al.*, 1959). Following 65 weeks of exposure, one of six animals developed a tumor during a test period of 84 weeks. These results indicate that the benzyl side chain of penicillin G contributes to carcinogenicity since the 6-aminopenicillanic acid does not have the side chain and appears to be somewhat less potent as a tumor-inducing agent.

D. Six-Membered Carcinogenic Lactones

d-Parasorbic acid (Fig. 8) is a naturally occurring α,β-unsaturated six-membered lactone which demonstrates a differential effect on inhibition of cells in tissue culture (Medawar *et al.*, 1943). The compound blocks the growth of fibroblasts without inhibiting epithelial cells (Medawar *et al.*, 1943; A. R. Powell, 1966). A similar form of cell type specificity has been observed in tissue-cultured cells exposed to aflatoxin (Terao, 1967; Terao and Miyaki, 1968; Zuckerman *et al.*, 1968). Further evaluation of the biological activity of parasorbic acid demonstrated that it was carcinogenic (Dickens and Jones, 1963a; Van der Merwe *et al.*, 1965). Administration

d-Parasorbic acid

5-Oxo-5*H*-benzo [*e*] isochromeno [4.3*b*] indole



Alternariol

Citreoviridin

FIG. 8. Six-membered carcinogenic lactones and related structures.

of 2 mg doses subcutaneously twice weekly for 32 weeks to rats induced formation of tumors in four or five animals during a 61-week test period.

Although structural analogs of parasorbic acid synthesized by molds have not been characterized as carcinogens, *Aspergillus nidulans* and a *Nigrospora* sp. produce α,β-unsaturated six-membered lactones (Argoudelis and Zieserl, 1966; Evans *et al.*, 1969). These fungal products are biologically active, demonstrating antibiotic activity. Other molds elaborate compounds containing six-membered lactones attached to various cyclic structures (fuscin, hispidin, geodoxin, rotiorin, and xylindein) (Shibata *et al.*, 1964). The metabolites of dermatophytic *Trichophyton* species have been extensively studied (Ng and Just, 1969; Shibata *et al.*, 1964). Elucidation of the structure of three of these substances—xanthomegnin, vioxanthin, and viopurpurin—has shown that they contain six-membered lactones fused to bicyclic moieties.

Penicillium toxicarum, *Penicillium ochrosalmoneum*, and *Penicillium citreoviride* produce a yellow fluorescent polyene, citreoviriden (Fig. 8), which contains an α,β-unsaturated six-membered lactone structure (Sakabe *et al.*, 1964). The substance in animals localizes in the central nervous system, affecting the spinal cord and medulla oblongata, resulting in respiratory paralysis. The minimum lethal dose for rats is 8 to 30 mg/kg, depending on the route of administration.

Another group of lactones has been implicated in carcinogenesis as a

result of work by Buu-Hoi *et al.* (1966) and Lacasagne *et al.* (1967) with 5-oxosulfhydrylbenzo(*e*)isochromeno(4,3-b)indole (Fig. 8). A 0.6-mg dose of this substance was injected subcutaneously into the flanks of mice three times, with a month between injections. Of the 54 mice tested, 52 developed sarcomas, and tumors were detected as early as 78 days following the first injection. Since the carcinogen contains an isocoumarin moiety, the workers warn against the hazard of naturally occurring compounds with similar structures. Substituted isocoumarins are common fungal metabolites produced by several species of *Aspergillus, Marasmius, Sporormia,* and *Pestalotia* (Shibata *et al.*, 1964). Ochratoxin (*A. ochraceus*), a known hepatoxin (Van der Merwe *et al.*, 1965), contains an isocoumarin moiety as does alternariol (*Alternaria tenuis*) (Fig. 8), a compound that interferes with normal cellular development (Raistrick *et al.*, 1953; Specter, 1957). Another group of biologically active isocoumarins, isolated from *Oospora astringenes*, are skin irritants and induce contraction of the tracheal muscle of guinea pigs (Nitta *et al.*, 1963). Although no isocoumarin derivative of natural origin has been shown to be carcinogenic, tumor initiation by the synthetic compound (Buu-Hoi *et al.*, 1966) and the hepatoxicity of ochratoxin indicate that this group of fungal products must be systematically analyzed for deleterious effects in animals.

Since aflatoxin contains a lactone in the coumarin nucleus and because many other compounds containing coumarin are biologically active (Soine, 1964), the toxicity and carcinogenicity of structures of this type are being reconsidered. An interesting group of structural analogs of aflatoxins are the furocoumarins. Scheel (1967) and Scheel *et al.* (1963) isolated from celery infected with *Sclerotinia sclerotiorum* two compounds identified as 4,5′,8-trimethylpsoralen and 8-methoxypsoralen. The compounds were not detected in nondiseased celery. Whether the compounds are produced by the parasite or the plant in response to the parasite is not known. Some furocoumarins are highly active photosensitizing agents capable of inducing sunburn and augmenting skin pigmentation (Musajo and Rodighiero, 1962). The pink rot dermatitis syndrome (Scheel *et al.*, 1963), a blistering cutaneous disorder affecting humans coming into contact with plants infected with *S. sclerotiorum*, could be the result of the action of the furocoumarins. Some of the substituted coumarins are also antibiotics, antiviral agents, and mutagens (Colombo *et al.*, 1965; Fowden *et al.*, 1967; Martin *et al.*, 1966; Mathews, 1963).

Van Duuren and co-workers (1963, 1965, 1966, 1967a,b; Van Duuren, 1965; Van Duuren and Goldschmidt, 1966) have compared the carcinogenicity of many epoxides, peroxides, and lactones. They found that β-butyrolactone and 2,2,4-trimethyl-3-hydroxy-3-pentenoic acid-β-lactone (Fig. 7) initiated tumor formation in test animals. Furthermore, several

epoxy and peroxy compounds were tumor initiators. Although several microbial metabolites contain the epoxy group (Shibata *et al.*, 1964), the scirpenes (sesquiterpenoid spiroepoxy compounds) have received the greatest attention as mycotoxins (Bamburg *et al.*, 1968; Guarino *et al.*, 1968). No definitive evidence has been presented implicating naturally occurring epoxy compounds as tumor initiators. However, the requirement for an intact epoxy group in the scirpenes for biological activity indicates that *in vivo* alkylation is involved in the toxic process. The toxicity of the naturally occurring scirpenes toward animals, plants, and microbes together with the Van Duuren studies suggests that further studies are required to evaluate properly the hazards of this group of natural products.

Since a large number of the tests for carcinogenesis have used subcutaneous injection into rodents, it should be mentioned that the validity of this method has been questioned (Clayson, 1962). However, support for this technique has also been presented (Hueper and Conway, 1964) and recent work by Van Duuren *et al.* (1963, 1965, 1966) reinforces its credibility. In their experiments, comparisons were made of a group of compounds administered in various ways to mice and rats. Examination of 12 epoxides, lactones, and peroxy compounds by skin tests, subcutaneous injection, and intragastric feeding demonstrated a striking correlation in tumor initiation in the three test systems by any one of the compounds.

E. Other Biologically Active Lactones

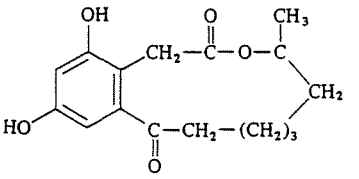
Several other fungal metabolites containing a lactone function are of interest because of their biological activity or structural similarity to carcinogenic lactones. Mycophenolic acid, a product of several *Penicillium* species, contains a five-membered lactone ring fused to a benzene moiety (Birkinshaw *et al.*, 1952; Shibata *et al.*, 1964). The compound exhibits toxic properties toward bacteria, fungi, and viruses, and is an effective antitumor agent (Cline *et al.*, 1969; Gilliver, 1946; Williams *et al.*, 1968). Other substances including cyclopolic-cyclopaldic acids and 7-hydroxy-4,6-dimethylphthalide are mold metabolites that are structurally similar to mycophenolic acid (Shibata *et al.*, 1964).

Tricothecium roseum produces a group of diterpenes containing a lactone structure, including rosolactone, rosenonolactone, deoxorosenonolactone, and 6β-hydroxyrosenonolactone (Holzapfel and Steyn, 1968). In studies of toxigenic fungi isolated from foodstuffs, Holzapfel and Steyn (1968) isolated a strain of the mold that elaborated substances toxic to ducklings. They attributed the toxicity to the cumulative effect of rosolactone, ergosterol, and 6β-hydroxyrosenonolactone. Similar types of

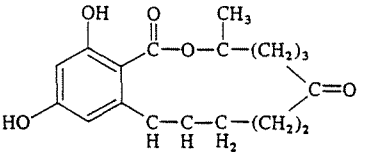
lactones are elaborated by *Claviceps purpurea* (Ergoflavin, Ergochrysin) (Shibata *et al.*, 1964). Furthermore, many of the gibberellin or giberellin-like compounds produced by *Giberella* strains are terpenoids containing lactone structures (Shibata *et al.*, 1964). The biological action of this group of compounds as plant growth regulators has been studied extensively.

An estrogenic response in swine consuming moldy corn has been attributed to substances produced by *Fusarium graminearum* and *Fusarium moniliforme* (Andrews and Stob, 1965; Stob *et al.*, 1962). One of these compounds has been isolated and characterized as 6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcylic acid (Fig. 9) (Christensen *et al.*, 1965; Mirocha *et al.*, 1967); its trivial name is zearalenone. Substances structurally similar but differing in biological activity are synthesized by various molds, including radiciol (monorden), *Nectria radicicola* (McCapra *et al.*, 1964; Mirrington *et al.*, 1964); and curvularin, *Penicillium expansum*, *Penicillium steckii*, and *Curvularia* sp. (Birch *et al.*, 1959; Shibata *et al.*, 1964). The structures of zearalenone and curvularin are presented in Fig. 9 to demonstrate the striking similarity of the two compounds. Similar macrocyclic lactones are produced by *Chaetomium funicola*, *Colletotrichum capaici* (Colletodiol) (MacMillan and Pryce, 1968; J. W. Powell and Whalley, 1969); *Torulopsis apicola* (di-*O*-acetyl-sophoroside) (Tulloch *et al.*, 1967); *Pithomyces chartarum* (Sporidesmolide I) (Shibata *et al.*, 1964); and *Fusarium* spp. and *Gibberella* spp. (Enniatin A-B) (Shibata *et al.*, 1964).

Another group of compounds that contain lactone structures are the verrucarins and muconomycins. These products of *Myrothecium verru-*



Curvularin



Zearalenone

FIG. 9. Structure of zearalenone and curvularin.

caria contain a sesquiterpenoid skeleton with an epoxy group and a macrocyclic triester lactone (Bamburg *et al.*, 1968; Guarino *et al.*, 1968). The substances are very toxic, initiating inflammation in the peritoneal cavity of rats and symptoms of creatinuria. The macrocylcic lactones (macrolides) produced by *Actinomycetes* have received a great deal of attention since several compounds of this type are effective antibiotics.

Other large complex molecules synthesized by molds also contain lactone functions. The ergot alkaloid ergosecaline contains a six-membered lactone (Shibata *et al.*, 1964), whereas the mycotoxin implicated in alimentary toxic aleukia, sporofusariogenin, is a steroid containing a six-membered lactone (Joffee, 1965; Olifson, 1960). A substance with a similar structure, antheridiol, produced by *Achlya bisexualis* has been identified as a fungal sex hormone (Edwards *et al.*, 1969).

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